

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representation of
The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

United States Patent [19]

Kossovsky et al.

US005219577A

[11] Patent Number: 5,219,577

[45] Date of Patent: Jun. 15, 1993

[54] BIOLOGICALLY ACTIVE COMPOSITION HAVING A NANOCRYSTALLINE CORE

[75] Inventors: Nir Kossovsky, Los Angeles; Rointan F. Bunshah, Playa del Rey, both of Calif.

[73] Assignee: The Regents of the University of California, Oakland, Calif.

[21] Appl. No.: 542,255

[22] Filed: Jun. 22, 1990

[51] Int. Cl.³ A61K 9/16

[52] U.S. Cl. 424/494; 424/9;
424/88; 424/89; 424/490; 424/493; 514/770;
514/951; 514/970

[58] Field of Search 424/494, 489, 490, 493,
424/497

[56] References Cited

U.S. PATENT DOCUMENTS

4,225,581 9/1980 Kreuter et al. 424/88
4,329,332 5/1982 Couvreur et al. 424/9
4,744,760 5/1988 Molday 44/493

OTHER PUBLICATIONS

Hayashi, C., Ultrafine Particles, J. Vac. Sci. Technol. A5(4), Jul./Aug. 1987 pp. 1375-1384.
Eastman, J. et al., Nanophase Synthesis Assembles Materials From Atomic Clusters, Res. & Dev. Jan. 1989, pp. 56-60.
Matijevic E., Fine Particles Part II, MRS Bulletin, Jan. 1990, pp. 16-49.
Kvalheim, G., Immunomagnetic Removal of B-Lymphoma Cells . . . , Bone Marrow Transplantation (1989), 4, pp. 567-574.
Varga, J. M., Immobilization of Small Molecules and Proteins. . . , FASEB Journal, vol. 4 Jun. 1990, pp. 2671-2677.
Juliano, R. L. (Ed) Biological Approaches to the Controlled Delivery of Drugs, Anals of NY Acad. Sci. vol. 507, pp. 104-141, pp. 211-219 and 252-280.
Faulk, et al., An Immunocolloid Method for the Electron Microscope, Immunochemistry, vol. 8, 1971, pp. 1081-1083.

Hainfeld, J. F., Gold Cluster-Labelled Antibodies, Nature vol. 333, May 1988 pp. 281-282.

Harven, E. et al., A Novel Approach for SEM of Coll. Goldlabelled Cell Surfaces, Journ. of Cell Biol., vol. 99, Jul. 1984, pp. 53-57.

Hsu, Y., Immunogold for Detection of Antigen on Nitrocellulose paper, Anal. Bioch., 142, 221-225 (1984).

Randall, R. E., Solid Matric-antibody-antigen . . . , Immunology Today, vol. 10, No. 10, 1989 pp. 336-339.
Johansson, M. E. et al., Prep. of Specific Antisera Against Adenoviruses . . . , Journ. of Immun. Methods, 26(1979) 141-149.

Warren, H. S. et al., Current Status of Immunological Adjuvants, Ann. Rev. Immunol. 1986 4:369-388.

Davis, S. S., Polymeric Microspheres as Drug Carriers, Biomaterials, 1988, vol. 9, Jan. pp. 111-115.

Zymed Brochure-Immunochemical Products-1989.
Advanced Magnetics, Inc. Brochure-Research Products 1988-1989.

(List continued on next page.)

Primary Examiner—Thurman K. Page

Assistant Examiner—James M. Spear

Attorney, Agent, or Firm—Poms, Smith, Lande & Rose

[57]

ABSTRACT

A biologically active composition made up of core particles having diameters of less than about 1000 nanometers which are coated with a layer which is designed to allow attachment of biologically active proteins, peptides or pharmacological agents to the microparticles. When viral protein is attached to the core particles, the result is a viral decoy which accurately mimics the native virus in both size and structure while being entirely devoid of virulent activity due to the microparticle core. Other antigenic proteins or peptides are attached to provide molecules which are useful in raising antibodies or as a diagnostic tool. Further, pharmacological agents are attached to the microparticles to provide pharmaceutical compositions.

13 Claims, No Drawings

OTHER PUBLICATIONS

Dynal AS Brochure—Immunomagnetic Separations Using DYNABEADS®—undated.

Kubiak, C., Couvreur, P., Manil, L., Clausse, B., "Increased cytotoxicity of nanoparticle-carried Adriamycin in vitro and potentiation by verapamil and amiodarone", *Biomaterials* 1989, vol. 10, Oct. pp. 553-556.

Manil, L., Roblot-Treupel, L., Couvreur, P., "Isobutyl cyanoimmunoradiometric assay", *Biomaterials* 1986, vol. 7 May 1986, pp. 212-216.

Couvreur, P., Kante, B., Lenaerts, V., Scailteur, V., Roland, V., and Speiser, P., "Tissue Distribution of Antitumor Drugs Associated with Polyalkylcyanoacrylate Nanoparticles", *Jour. of Pharm. Sci.* vol. 69, No. 2, Feb. 1980, pp. 199-202.

Couvreur, P., Kante, B., Roland, M., Guio, P., Bauduin, P., Speiser, P. "Communications", *J. Pharm. Pharmacol.* 1979, 31:331-332.

Pyle, Stephen W., et al., "Immune response to immunostimulatory complexes . . .", *Vaccine*, vol. 7, Oct. 1989, pp. 465-473.

Lovgren, Karin, and Morein, Bror, "The Iscom: An Antigen Delivery System with Built-In Adjuvant", *Molecular Immun.*, vol. 28, No. 3, pp. 285-286, 1991.

Morein, B., "Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses", *Letters to Nature*, Mar. 1984, pp. 457-460.

Morein, Bror, "The iscom: an Immunostimulating System", *Immunology Letters*, 25 (1990) pp. 281-284.

BIOLOGICALLY ACTIVE COMPOSITION HAVING A NANOCRYSTALLINE CORE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to a synthetic biologically active composition having a microparticulate core. More particularly, the present invention relates to synthetic, biologically active compositions comprising at least one biologically active peptide, protein or pharmacologic agent attached to a nanocrystalline core particle. The invention further relates to methods of using the resulting synthetic compositions as vaccines, immunodiagnosics or as pharmaceuticals, depending upon the nature of the particular biologically active moiety.

2. Description of Related Art

The attachment of biologically active proteins, peptides or pharmacologic agents to various carrier particles has been an area of intense investigation. These conjugated biological systems offer the promise of reduced toxicity, increased efficacy and lowered cost of biologically active agents. As a result, many different carrier models are presently available. (Varga, J. M., Asato, N., in Goldberg, E. P. (ed.): *Polymers in Biology and Medicine*. New York, Wiley, 2, 73-88 (1983). Ranney, D. F., Huffaker, H. H., in Juliano, R. L. (ed.): *Biological Approaches to the Delivery of Drugs*, Ann. N.Y. Acad. Sci., 507, 104-119 (1987).) Nanocrystalline and micron sized inorganic substrates are the most common carriers and proteins are the most commonly conjugated agents. For example, gold/protein (principally immunoglobulin) conjugates measuring as small as 5 nm have been used in immunological labeling applications in light, transmission electron and scanning electron microscopy as well as immunoblotting. (Faulk, W., Taylor, G., *Immunochimistry* 8, 1081-1083. (1971). Hainfeld, J. F., *Nature* 333, 281-282 (1988).)

Silanized iron oxide protein conjugates (again principally antibodies) generally measuring between 500 and 1500 nm have proven useful in various in vitro applications where paramagnetic properties can be used advantageously. (Research Products Catalog, Advanced Magnetism, Inc., Cambridge, MA, 1988-1989.) Ugelstad and others have produced gamma iron oxides cores coated with a thin polystyrene shell. (Nustad, K., Johansen, L., Schmid, R., Ugelstad, J., Ellengsen, T., Berge, A.: Covalent coupling of proteins to monodisperse particles. Preparation of solid phase second antibody. *Agents Actions* 1982; 9:207-212 (id. no. 60).) The resulting 4500 nm beads demonstrated both the adsorption capabilities of polystyrene latex beads as well as the relatively novel benefit of paramagnetism.

Carrier systems designed for in vivo applications have been fabricated from both inorganic and organic cores. For example, Davis and Illum developed a 60 nm system comprised of polystyrene cores with the block copolymer poloxamer, polyoxyethylene and polyoxypropylene, outer coats that showed a remarkable ability to bypass rat liver and splenic macrophages. (Davis, S., Illum, L., *Biomaterials* 9, 111-115 (1988).) Drug delivery with these particles has not yet been demonstrated. Ranney and Huffaker described an iron-oxide/albumin/drug system that yielded 350-1600 nm paramagnetic drug carriers. (Ranney, D. F., Huffaker, H. H., in Juliano, R. L. (ed.): *Biological approaches to the delivery of drugs*, Ann. N.Y. Acad. Sci. 507, 104-119

(1987).) Poznasky has developed an enzyme-albumin conjugate system that appears to decrease the sensitivity of the product to biodegradation while masking the apparent antigenicity of the native enzyme. (Poznasky, M. J.: Targeting enzyme albumin conjugates. Examining the magic bullet. In, Juliano, R. L. (ed.): *Biological approaches to the delivery of drugs*, Annals New York Academy Sciences 1987; 507-211:219.)

Shaw and others have prepared and characterized lipoprotein/drug complexes. (Shaw, J. M., Shaw, K. V., Yanovich, S., Iwanik, M., Futch, W. S., Rosowsky, A., Schook, L. B.: Delivery of lipophilic drugs using lipoproteins. In, Juliano, R. L. (ed.): *Biological approaches to the delivery of drugs*, Annals New York Academy Sciences 1987; 507:252-271.) Lipophilic drugs are relatively stable in these carriers and cell interactions do occur although little detail is known.

In any conjugated biological composition, it is important that the conformational integrity and biological activity of the adsorbed proteins or other biological agents be preserved without evoking an untoward immunological response. Spatial orientation and structural configuration are known to play a role in determining the biological activity of many peptides, proteins and pharmacological agents. Changes in the structural configuration of these compounds may result in partial or total loss of biological activity. Changes in configuration may be caused by changing the environment surrounding the biologically active compound or agent. For example, pharmacologic agents which exhibit in vitro activity may not exhibit in vivo activity owing to the loss of the molecular configuration formerly determined in part by the in vitro environment. Further, the size and associated ability of the carrier particle to minimize phagocytic trapping is a primary concern when the composition is to be used in vivo. All of these factors must be taken into account when preparing a carrier particle.

Although numerous different carrier particles have been developed, there is a continuing need to provide carrier particles for both in vivo and in vitro application wherein a biologically active peptide, protein or pharmacological agent can be attached to the particles in a manner which promotes stabilization of the biologically active compound in its active configuration. The present invention relates to such particles and compositions.

SUMMARY OF THE INVENTION

In accordance with the present invention, biologically active peptides, proteins or pharmacological agents are attached to a core particle to provide a wide variety of biologically active compositions. The invention is based on the discovery that the surface of ultrafine particles (nanocrystalline particles) can be modified with a surface coating to allow attachment of biologically active moieties to produce compositions wherein the naturally occurring structural environment of the moiety is mimicked sufficiently so that biological activity is preserved. The coating which provides for the attachment of biologically active moieties to nanocrystalline particles in accordance with the present invention is composed of a basic or modified sugar or oligonucleotide. Coating nanocrystalline particles with a basic sugar or oligonucleotide produces changes in the surface energy and other surface characteristics which make the particles well suited for attachment of biologically active moieties.

In one embodiment of the present invention, nanocrystalline particles are used to prepare a decoy virus wherein the DNA or RNA core of the virus is replaced by the microparticle. The microparticle is chosen to be the same size as the viral core so that the conformation of the surrounding protein coat accurately mimics the native virus. The resulting viral decoy is incapable of infectious behavior while at the same time being fully capable of effecting an immune response and otherwise being antigenically bioreactive.

In this embodiment, an ultrafine particle having a diameter of less than about 1000 nanometers is chosen so as to mimic the DNA or RNA core. Viral peptides attached to the coating surrounding the core have a structure which mimics at least a portion of the native virus. This size of microparticle core is also well suited for carrying anchorage dependent pharmacological agents and other biologically active compounds which require a nanocrystalline particle anchor or core in order to maintain their activity.

Nanocrystalline particles suitable for use in the present invention can be made from metals, ceramics, or polymers. Examples of appropriate materials include chromium, rubidium, iron, zinc, selenium, nickel, gold, silver, platinum, silicon dioxide, aluminum oxide, ruthenium oxide, tin oxide and polystyrene.

The biologically active microparticles in accordance with the present invention have wide-ranging use depending upon the type of biologically active compound which is attached to the microparticle core. When viral protein is attached to the microparticle core, the result is a decoy virus which may be used as a vaccine, diagnostic tool or antigenic reagent for raising antibodies. Non-viral protein or antigen coatings may be selected and structured for use in raising specific antibodies or as a diagnostic tool. Further, the microparticles can function as a pharmacological agent when compounds having pharmacological activity are attached to the core particle.

In accordance with the present invention, the utilization of a core microparticle around which the viral protein is attached provides an effective way to accurately mimic the antigenic reactivity of a native virus while totally eliminating any of the problems and risks associated with the presence of the viral genetic material. In addition, other proteins, peptides or pharmacological agents may be attached to the core particle to preserve and/or enhance the activity of the compound.

The above-discussed and many other features and attendant advantages of the present invention will become better understood by reference to the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention has wide application to immunologic procedures and methods wherein antigenic material or other biologically active moieties are utilized. These areas of application include vaccination agents, antigen agents used to raise antibodies for subsequent diagnostic uses and antigenic compounds used as diagnostic tools. The composition of the invention can also be used in a wide variety of other applications where there is a need to anchor a protein, peptide or pharmacological agent to a core particle in order to preserve and/or enhance bioreactivity.

The compositions of the present invention include nanocrystalline core particles (diameters of less than

1000 nm) which are coated with a surface energy modifying layer that promotes bonding of proteins, peptides or pharmaceutical agents to the particles. The coating modifies the surface energy of the nanocrystalline core particles so that a wide variety of immunogenic proteins, peptides and pharmaceutical agents may be attached to the core particle without significant loss of antigenic activity or denaturation. The result is a biologically active composition which includes a biologically inert core. The end use for the compositions of the present invention will depend upon the particular protein, peptide or pharmacological agent which is attached to the coated core particle. For example, proteins or peptides having antigenic activity may be attached to provide compositions useful as immunodiagnostic tools. Viral fragments or protein coatings having immunogenic activity may be attached to provide a vaccine. Also, pharmacological agents may be attached to provide compositions which are useful in treating diseases.

For preparing decoy viruses for use as vaccines, particles having diameters of between about 10 to 200 nanometers are preferred since particles within this size range more closely mimic the diameter of DNA and RNA cores typically found in viruses.

The core particles may be made from a wide variety of inorganic materials including metals or ceramics. Preferred metals include chromium, rubidium, iron, zinc, selenium, nickel, gold, silver, platinum. Preferred ceramic materials include silicon dioxide, aluminum oxide, ruthenium oxide and tin oxide. Preferred polymers include polystyrene, nylon and nitrocellulose. Particles made from tin oxide, silicon dioxide or gold are particularly preferred.

Particles made from the above materials having diameters less than 1000 nanometers are available commercially or they may be produced from progressive nucleation in solution (colloid reaction), or various physical and chemical vapor deposition processes, such as sputter deposition (Hayashi, C., *J. Vac. Sci. Technol.* A5 (4), July/August 1987, pgs. 1375-1384; Hayashi, C., *Physics Today*, December 1987, pgs. 44-60; *MRS Bulletin*, January 1990, pgs. 16-47). Tin oxide having a dispersed (in H₂O) aggregate particle size of about 140 nanometers is available commercially from Vacuum Metallurgical Co. (Japan). Other commercially available particles having the desired composition and size range are available from Advanced Refractory Technologies, Inc. (Buffalo, N.Y.).

Plasma-assisted chemical vapor deposition (PACVD) is one of a number of techniques that may be used to prepare suitable microparticles. PACVD functions in relatively high atmospheric pressures (on the order of one torr and greater) and is useful in generating particles having diameters of up to 1000 nanometers. For example, aluminum nitride particles having diameters of less than 1000 nanometer can be synthesized by PACVD using Al(CH₃)₃ and NH₃ as reactants. The PACVD system typically includes a horizontally mounted quartz tube with associated pumping and gas feed systems. A susceptor is located at the center of the quartz tube and heated using a 60 KHz radio frequency source. The synthesized aluminum nitride particles are collected on the walls of the quartz tube. Nitrogen gas is used as the carrier of the Al(CH₃)₃. The ratio of Al(CH₃)₃:NH₃ in the reaction chamber is controlled by varying the flow rates of the N₂/Al(CH₃)₃ and NH₃ gas into the chamber. A constant pressure in the reaction

chamber of 10 torr is generally maintained to provide deposition and formation of the ultrafine nanocrystalline aluminum nitride particles. PACVD may be used to prepare a variety of other suitable nanocrystalline particles.

The core particles are coated with a substance that provides a threshold surface energy to the particle sufficient to cause binding to occur without that binding being so tight as to denature biologically relevant sites. Coating is preferably accomplished by suspending the particles in a solution containing the dispersed surface modifying agent. It is necessary that the coating make the surface of the particle more amenable to protein or peptide attachment. Suitable coating substances in accordance with the present invention include cellobiose, related basic sugars, and modified sugars such as nitrocellulose. Oligonucleotides may also be used. Suitable oligonucleotides include polyadenosine (polyA). Cellobiose is a preferred coating material.

The coating solution into which the core particles are suspended contains, for example, from 1 to 30 weight/volume percent of the coating material. The solute is preferably double distilled water (ddH₂O). The amount of core particles suspended within the coating solution will vary depending upon the type of particle and its size. Typically, suspensions containing from 0.1 to 10 weight/volume percent are suitable. Suspensions of approximately 1 weight/volume percent of particles are preferred.

The core particles are maintained in dispersion in the coating solution for a sufficient time to provide uniform coating of the particles. Sonication is the preferred method for maintaining the dispersion. Dispersion times ranging from 30 minutes to a few hours at room temperature are usually sufficient to provide a suitable coating to the particles. The thickness of the coating is preferably less than 5 nanometers. Thicknesses of the coating may vary provided that the final core particles include a uniform coating over substantially all of the particle surface.

The particles are separated from the suspension after coating and may be stored for future use or redispersed in a solution containing the protein or peptide to be attached to the particles. Alternatively, the coated particles may be left in the suspension for further treatment involving attachment of the desired protein or peptide.

The protein or peptide which is applied to the coated particles may be selected from a wide variety of proteins or peptides. Those having antigenic properties are preferred when a vaccine is required. The protein can be the viral protein coat from a selected virus or immunogenic portion thereof. The viral protein coat is isolated according to known separation procedures for isolating and separating viral proteins. The viral coating is the preferred protein because the viral coating is where the antigenic activity of viruses is known to be located. Typically, the virus is digested or solubilized to form a mixture of viral proteins. The viral proteins are then separated by liquid chromatography or other conventional process into the various protein particle fractions and dialyzed to remove impurities.

Suitable viruses from which viral protein particles can be separated and isolated include Epstein-Barr virus, human immunodeficiency virus (HIV), human papilloma virus, herpes simplex virus and pox-virus. Preparations of a wide variety of antigenic protein materials may also be purchased commercially from supply houses such as Microgene Systems, Inc. (400 Frontage

Road, West Haven, Conn. 06516), Amgen Corporation (1900 Oak Terrace Lane, Thousand Oaks, Calif. 91320-1789) and Cetus Corporation (1400 53rd Street, Emeryville, Calif. 94608).

Other biologically active proteins and peptides that can be attached include enzymes, hormones, transport proteins and protective proteins. Human serum transferrin, plasminogen activator and coagulation factors, in addition to the pharmacologic agents amphotericin and insulin, are examples.

The procedure for attaching the antigens or other protein to the coating on the core particles involves suspending the coated core particles in an aqueous solution containing the antigen. The presence in the solution of materials that may preferentially attach to the particle surface is often not advantageous. For example, the dispersion agents present in the solution may create an undesirable coating on the suspended particles prior to protein attachment. Water miscible solvents such as methanol or ethanol may be used. The aqueous solution of coated microparticles can be agitated sufficiently to provide a uniform suspension of the particles. Typically, the amount of particles in solution will be between about 0.5 mg per milliliter of solution and 5 mg per milliliter of solution. Sonication is a preferred method for providing a uniform suspension of the coated particles in solution.

The suspension of coated particles and antigens must be within certain parameters for protein attachment and assembly to occur. The temperature of the particle solution should be between 30° C. to 45° C. Certain proteins and pharmaceutical agents may be bound to the coated particles in distilled water. Salts may be added to the solution for reactions between coated particles and proteins and other pharmaceutical agents which are unstable or will not disperse readily in distilled water. In general, the salt solutions should be formulated so that the ionic balance (in mM) does not exceed: K=300-500; Na=30-70; Cl=40-150; Ca=0.0003-0.001; and Mg=0.0003-0.001. The oxygen tension of the solution is, advantageously, less than 10% in a solution sparged initially by helium and then gassed with helium, nitrogen and carbon dioxide. The pH of the solution is, advantageously, slightly acidic (relative to blood), with a value, preferably, of between 6.8 to 7.2. An exemplary solution for dispersion of the coated microparticles and for protein attachment is an aqueous solution containing: 0.0360 milligrams MgSO₄ per liter, 0.0609 milligrams MgCl₂·6H₂O, 0.0441 milligram CaCl₂·2H₂O, 22.823 grams K₂HPO₄, 13.609 grams KH₂PO₄, 7.455 grams KCl, and 4.101 gram sodium acetate. The pH of this solution is adjusted to 6.8.

The coated particle cores with the attached protein can be separated from the ionic growth medium and stored for further use. The coated particles may be stored by any of the conventional methods typically used for storing antigenic compounds or antibodies. For example, the coated particles may be freeze dried or stored as a suspension in a compatible solution. When used as a vaccine, the particles coated with a viral protein coat are injected or otherwise administered to the individual according to conventional procedures. Any pharmaceutically acceptable carrier solution or other compound may be used in administering the coated particles to the individual. When used for diagnostic purposes in vitro, the protein coated particles are suspended in solution and used in the same manner as other antigenic compounds. The same is true for use of the

protein coated particles for raising antibodies. The same protocol and procedures well known for using antigens to produce antibodies may be used wherein the protein coated particles of the present invention are substituted for normally used antigenic compounds.

The following non-limiting examples describe certain aspects of the present invention in greater detail.

EXAMPLE 1

Preparation of nanocrystalline tin oxide microparticles

1.5 to 2.0 mg of ultrafine (nanocrystalline) metal powder was placed in a 1.7 ml screw-cap microcentrifuge with 1.5 mls of double distilled water (ddH₂O). The ddH₂O was filtered through a rinsed 0.45 micron filter-sterilizing unit or acrodisc (Gelman Scientific). The metal powder was tin oxide with a mean diameter (by photon correlation spectroscopy) of 140 nm. The mixture was vortexed for 30 seconds and placed into a water sonicating bath overnight. The sonication bath temperature was stabilized at 60° C. After a 24-hour sonication, the samples were vortexed once more for 30 seconds with the resulting dispersion clarified by microcentrifugation at approximately 16,000 rpm for 15 seconds. The analysis of particle size was carried out on a Coulter N4MD submicron particle analyzer.

The coating was applied to the tin oxide particles by suspending the particles in a stock solution of cellobiose. The cellobiose stock solution was a 292 mM solution made by dissolving 1.000 gram of cellobiose in 9.00 ml of ddH₂O. Solution was accomplished at approximately 70° C. in order to promote quick dissolution. The resulting cellobiose solution was filter sterilized through a rinsed 0.45 micron filter with the final volume being adjusted to 10.00 ml.

Sufficient cellobiose stock solution was added to 150 microliters of ultrafine tin oxide dispersion so that the final concentration of the tin oxide was 1.00 percent (w/v) or 29.2 mM. A typical volume for preparation was 2.0 mls which was mixed four or five times by the action of a micro-pipetor. After mixing, the dispersion was allowed to equilibrate for two hours. Demonstration of successful coating of the particles was provided by measuring the mobility of the particles (coated and uncoated) on a Coulter DELSA 440 doppler energy light scatter analyzer. The coated tin oxide particles exhibited a relatively low mobility compared to the non-coated tin oxide particles. Measurements were also taken at various dilute salt concentrations to ensure that the observations with respect to mobility were not artifactual. The tests demonstrate that the particles were coated with the cellobiose.

The coated particles are then used to attach antigenic proteins, peptides or pharmacological agents to prepare bioreactive particles.

EXAMPLE 2

Preparation of nanocrystalline ruthenium oxide particles

The same procedure was carried out in accordance with Example 1, except that ruthenium oxide microparticles were substituted for the tin oxide particles. The ruthenium oxide particles were obtained from Vacuum Metallurgical Company (Japan).

EXAMPLE 3

Preparation of the nanocrystalline silicon dioxide and tin oxide particles

Nanocrystalline silicon dioxide was acquired commercially from Advanced Refractory Technologies, Inc. (Buffalo, N.Y.) and tin oxide was acquired commercially from Vacuum Metallurgical Co. (Japan). The tin oxide particles were also prepared by reactive evaporations of tin in an argon-oxygen mixture and collected on cooled substrates. Nanocrystalline tin oxide was also synthesized by D.C. reactive Magnetron sputtering (inverted cathode). A 3" diameter target of high purity tin was sputtered in a high pressure gas mixture of argon and oxygen. The ultrafine particles formed in the gas phase were collected on copper tubes cooled to 77° K. with flowing liquid nitrogen. All materials were characterized by X-ray diffraction crystallography, transmission electron microscopy, photon correlation spectroscopy, and Doppler electrophoretic light scatter analysis. X-ray diffraction samples were prepared by mounting the powder on a glass slide using double-sized Scotch tape. CuK α radiation was used on a Norelco diffractometer. The spectrum obtained was compared with ASTM standard data of tin oxide. (Powder Diffraction File, Card #21-1250. Joint Committee on Power Diffraction Standards, American Society for Testing and Materials, Philadelphia 1976.) The specimens for (TEM) were collected on a standard 3 mm diameter carbon coated copper mesh by dipping into a dispersion of the (UFP's) in 22-propanol. The samples were examined on a JEOL-STEM 100 CX at an acceleration voltage of 60-80 KV.

To create working dispersions of these metal oxides, 1.5 to 3.0 mg of metal oxide powder was added to 1.5 ml double distilled H₂O in a dust-free screw top microcentrifuge tube (Sarsted) and vortexed for 30 seconds. The mixture was then sonicated for 16 to 24 hours followed by a second 30 seconds vortex. The submicron fraction was then isolated by pelleting macroparticulates by microcentrifugation 16,000 xg for 15 seconds. Approximately 1.3 ml of supernatant was then removed and placed in another dust-free screw top microcentrifuge tube. A sample was prepared for photon correlation spectroscopy (Coulter N4MD) and Doppler electrophoretic light scattering (Coulter delsa 440) analysis by removing 50 to 100 μ l of the dispersion and placing it in a polystyrene cuvette and diluting it to a final volume of 1.00 ml with ddH₂O. The stability of the dispersion was determined by sequential measurements over a 24-hour period and was found to be stable. The stability of the dispersion with respect to progressive salinity of the solvent (increasing conductivity) was similarly determined. The stability increased with progressive salinity of the solvent.

1.00 ml of the dispersion was combined and stirred with 8.00 ml of ddH₂O and 1.00 ml of 292 mM cellobiose stock in a 15.0 ml capacity ultrafiltration stir cell (Spectra) which has been fitted with a pre-rinsed 5 \times 10⁵ molecular weight cutoff type F membrane (Spectra). The sample was then left to stir for 15 minutes. After stirring, the excess cellobiose was removed by flushing through the cell chamber 250 ml of ddH₂O by the action of a peristaltic pump at a rate that does not exceed 10.0 ml/min. After washing, the filtrate was concentrated by the means of pressurized N₂ gas to approximately 1.0 ml. Character was established by the

removal of 500 μ l of the treated dispersion by N4MD analysis. The means dispersion diameter was re-established at this step. The stability of the coated dispersion was determined by sequential measurements over a 24-hour period. The stability of the coated dispersion with respect to progressive salinity of the solvent (increasing conductivity) was similarly determined.

The resulting coated nanocrystalline particles are suitable for attachment of various proteins, peptides and pharmaceutical agents.

EXAMPLE 4

Preparation, isolation and surface adsorption of human serum transferrin proteins

Nanocrystalline tin oxide was synthesized by D.C. reactive Magnetron sputtering (inverted cathode). A 3" diameter target of high purity tin was sputtered in a high pressure gas mixture of argon and oxygen. The ultra-fine particles formed in the gas phase were collected on copper tubes cooled to 77° K. with flowing liquid nitrogen. All materials were characterized by x-ray diffraction crystallography, selected area electron diffraction, transmission electron microscopy, photon correlation spectroscopy, and energy dispersive x-ray spectroscopy. X-ray diffraction samples were prepared by mounting the powder on a glass slide using double-sized Scotch tape. CuK(alpha) radiation was used on a Norelco diffractometer. The spectrum obtained was compared with ASTM standard data of tin oxide. The specimens for transmission electron microscopy and selected area diffraction were collected on a standard 3 mm diameter carbon coated copper mesh by dipping into a dispersion of the nanocrystalline materials in 2-propanol. The samples were examined on a JEOL-STEM 100 CX at an acceleration voltage of 60–80 KeV. The 2-propanol suspension of particles was also characterized by photon correlation spectroscopy at 22.5° C., 600 s run time on a Coulter N4MD. Energy dispersive x-ray spectroscopy was performed on a JEOL JSM-T330A scanning electron microscope using Kevex quantex V software.

To create working dispersions of these metal oxides for the synthesis of compositions in accordance with the present invention, 0.5 mg of metal oxide powder was added to 1.0 ml of a 292 mM cellobiose-phosphate buffered saline solution in a dust free screw top glass vial and sonicated for 20 minutes at 22.5°–35° C. The submicron fraction was then isolated by pelleting macroparticulates by microcentrifugation at 16,000 \times g for 30 seconds. Approximately 900 μ l of supernatant was then removed and placed in a dust free screw top microcentrifuge tube. An aliquot was removed for photon correlation spectroscopy (Coulter N4MD) and Doppler electrophoretic light scattering (Coulter DELSA 440) analysis. Aliquots were also removed for characterizing the stability of the coated dispersion over time and with respect to progressive salinity of the solvent (increasing conductivity).

To adsorb protein to the cellobiose coated metal oxide nanocrystalline cores, the core sample was diluted to 10.0 ml with Ca⁺⁺ and Mg⁺⁺ free phosphate buffered saline (Gibco). Forty (40.0) μ g of purified human serum transferrin (4 μ g/ μ l) (Gibco), whose antigenicity was verified by ELISA, was then added to a 10 ml stir cell (Spectra).

The sample was then left to stir slowly for 30 minutes, taking great care not to allow forming. After the addition period, 15 ml of Ca⁺⁺ and Mg⁺⁺ free phosphate

buffered saline (Gibco) was then washed through the cell under a 2 psi nitrogen gas pressure head. After washing, the sample was again concentrated to 1.00 ml under N₂ and a 500 μ l sample was removed for analysis by photon correlation spectroscopy, Doppler electrophoretic light scatter and transmission electron microscopy as detailed below.

Conformational integrity was assessed by measuring the retained antigenicity of the bound protein. To the sample cell, 50.0 μ l of rabbit polyclonal anti-human transferrin antibody (Dako), whose antigenicity was confirmed by ELISA, was added to the concentrated 1.0 ml reaction product at 37.5° C. with gentle stirring. After a 30 minute incubation period, 15 ml of Ca⁺⁺ and Mg⁺⁺ free phosphate buffered saline (Gibco) was then washed through the cell under a 2 psi nitrogen gas pressure head and the reaction volume was again reduced to 1.0 ml.

A 200 μ l aliquot of blocking agent, 1% w/v bovine serum albumin in divalent free saline, was added followed by a 10 minute equilibration period. The secondary antibody, 30 nm gold conjugated goat anti-rabbit polyclonal IgG (Zymed), was then added and the reaction mixture was allowed to incubate for 30 minutes. A sample was removed, chopped on a transmission electron microscopy grid, and vacuum dried. The mixture was again washed with 15 ml of divalent free saline under a nitrogen pressure head and then fixed with glutaraldehyde. One ml of 3% solid bovine collagen (Collagen Corp.) was then added to the mixtures and the composite was ultracentrifuged at 10⁶ \times g for 30 minutes yielding a pellet that was then routinely processed as a biological specimen for transmission electron microscopy. Ten nm thick sections were viewed on a Zeiss transmission electron microscopy. Control samples were prepared as above without the cellobiose intermediate bonding layer.

Transmission electron micrographs showed that the D.C. magnetron sputtered tin oxide was composed of individual particles measuring 20–25 nm in diameter which aggregated into clusters measuring 80 to 120 nm in diameter. By photon correlation spectroscopy, these same particles when dispersed in distilled water produced agglomerates measuring 154 \pm 55 nm. The tin oxide particles were fully crystalline as characterized by electron and x-ray diffraction. Energy dispersive x-ray spectroscopy showed no other elements present as impurities.

By Doppler electrophoretic light scatter analysis, tin oxide exhibited a mean mobility of 2.177 \pm 0.215 μ m-cm/V-s in aqueous solutions ranging from 10.8 to 20.3 μ M NaCl. Following cellobiose surface coating in a 1% solution, tin oxide exhibited a mean mobility of 1.544 \pm 0.241 μ m-cm/V-s in aqueous solutions ranging from 0.0 to 21.0 μ M NaCl. The oxide agglomerated in salt concentrations of greater than 40.0 μ M and in solutions of increasing cellobiose concentration.

Following transferrin binding, the crude tin oxide/cellobiose/protein conjugates measured 350 \pm 84 nm by photon correlation spectroscopy and transmission electron microscopy. Vacuum dried dropped samples with low concentration gold antibody measured 35–50 nm. Without the cellobiose bonding layer, vacuum dried sections measured 400 to >1000 nm. Occasional antibody bonding was noted. Following high concentration immunogold labeling and filtering, the thin section cellobiose treated specimens measured 50–100 nm. Positive

11

gold binding was identified in approximately 20% of the appropriately coated samples whereas negative controls (prepared as above but lacking the primary rabbit antibody) exhibited approximately 1% nonspecific binding.

As can be seen from the above examples, the biological activity of protein absorbed to the surface of carbohydrate-treated nanocrystalline metal oxide particles is preserved.

The entire contents of all references cited hereinabove are hereby incorporated by reference.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

What is claimed is:

1. A composition of matter comprising:
 - a core particle having a diameter of less than about 1000 nanometers, said core particle comprising a metal, ceramic or polymer;
 - a coating consisting essentially of cellobiose which at least partially covers the surface of said core particle, wherein said coating provides a threshold surface energy to said core particle which is sufficient to bind biologically active agents without denaturing said agents; and
 - at least one biologically active agent bound to said coated core particle wherein said biologically active agent bound to said coated core particle is not denatured.
2. A composition of matter according to claim 1 wherein the diameter of said core particle is between about 10 to 200 nanometers.
3. A coated particle for bonding biologically active agents thereto, wherein said coated particle comprises a core particle comprising a metal, ceramic or polymer, said core particle having a diameter of less than about

12

1,000 nanometers and a coating consisting essentially of cellobiose which at least partially covers the surface of said core particle wherein said coating provides a threshold surface energy to said core particles which is sufficient to bind biologically active agents to said core particle in a manner such that the biological activity of said agent is not denatured.

4. A composition of matter according to claim 1 wherein said biologically active agent is a viral peptide or protein.

5. A composition of matter according to claim 1 wherein said biologically active agent is an immunogenic peptide or protein.

6. A composition of matter according to claim 1 wherein said biologically active agent is a pharmacologic agent.

7. A coated particle according to claim 3 wherein the diameter of said core particle is between about 10 and 200 nanometers.

8. A composition of matter according to claim 1 wherein said metal is selected from the group consisting of chromium, rubidium, iron, zinc, selenium, nickel, gold, silver and platinum.

9. A composition of matter according to claim 1 wherein said ceramic is selected from the group consisting of silicon dioxide, aluminum oxide, ruthenium oxide and tin oxide.

10. A composition of matter according to claim 1 wherein said polymer is polystyrene.

11. A coated particle according to claim 3 wherein said metal is selected from the group consisting of chromium, rubidium, iron, zinc, selenium, nickel, gold, silver and platinum.

12. A coated particle according to claim 3 wherein said ceramic is selected from the group consisting of silicon dioxide, aluminum oxide, ruthenium oxide and tin oxide.

13. A coated particle according to claim 3 wherein said polymer is polystyrene.

* * * * *

45

50

55

60

65